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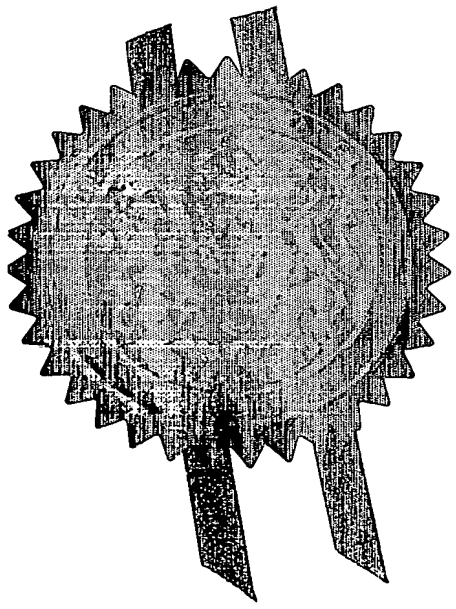

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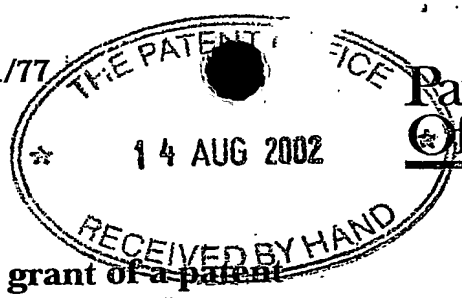


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ROSLIN INSTITUTE (EDINBURGH)
Roslin
Midlothian
EH25 9PS
GB

Patents ADP number *(if you know it)*

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6835912001

4. Title of the invention

AVIAN SEX DETERMINATION METHOD

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Kilburn & Strode
20 Red Lion Street
London
WC1R 4PJ

Patents ADP number *(if you know it)* — 125001

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Signature William J Strode — Date 14 August 2002

Kilburn & Strode

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# AVIAN SEX DETERMINATION METHOD

The present invention relates to a method for sexing individual subjects of avian species.

Adults and particularly offspring of many avian species are monomorphic, making determination of sex difficult. Nucleic acid probes that hybridise to the DNA of the female-specific *W* chromosome have lead to molecular solutions to this problem for some species. However, use of such techniques has proved to be difficult and in many cases, their taxonomic range is limited. More recently, polymerase chain reaction (PCR) based approaches that are technically simpler and that have broader taxonomic utility have been developed.

Sex identification methods have also been based upon examining differences in intron size between the female *W* specific chromosome and the *Z* chromosome, which occurs in both sexes (female, *ZW*; male, *ZZ*) (Ellegren 1996, Kahn *et al* 1998). Another approach has been to identify specific genes located on the *W* chromosome. Analysis of the chromobox-helicase-DNA-binding gene (CHD) shows that it contains sequences specific to the *W* chromosome and can be used for determination of the sex of most birds (Griffiths, *et al* 1998). A combination of the analysis of intron size difference between sexes and the chromosome specific CHD gene has also been proposed (Fridolfsson, A-K and Ellegren, H, 1999). Another method proposed has been to analyse W-specific repeat sequences in order to determine the sex of chick embryos (Clinton, 1994). However, this method required separate sexing and control PCR reactions. A rapid and simple single tube chicken sexing protocol based on a PCR analysis of *W* chromosome specific sequences has been devised more recently (Clinton *et al* 2001). Other methods have been proposed in WO 96/39505 based on an analysis of DNA sequences (introns and exons) encoding two genes located on the *Z* and *W* chromosomes of birds. Other means for analysis have been proposed in US-A-5,679,514 and US-A-5,707,809.

Such methods of sex determination generally require technical expertise and specialist facilities, and are not susceptible to ready automation, especially in agricultural environments. The sequences referred to also have variants (homologues in the case of genes) of that sequence present on the Z-chromosome (i.e. the avian sex chromosome that is found in both sexes).

The avian gene *WPKCI* has been shown to be conserved widely on the avian *W* chromosome and expressed actively in the female chicken embryo before the onset of gonadal differentiation. It is suggested that *WPKCI* may play a role in the differentiation of the female gonad by interfering with the function of PKCI or by exhibiting its unique function in the nucleus (Hori *et al* 2000). This gene has also been identified as *ASW* (avian sex-specific W-linked) (O'Neill *et al* 2000).

Accurate determination of the sex of avians is a particularly important issue for the poultry industry for both economic and welfare reasons. Companies which produce egg-layer strains of chickens ("layers") would prefer to be able to (inexpensively) determine the sex of birds at hatch and just raise the females; companies which produce meat strains of chickens ("broilers") would prefer to (inexpensively) sex birds at hatch and just raise males as they grow much faster and eat less. Currently, most "broiler" producers accept the inefficiency of producing and rearing female birds, whilst a proportion of the "layer" producers use relatively expensive procedures to determine the sex of one-day old chicks.

The use of sex determination procedures on one-day old chicks has significant welfare implications, in particular the disposal of unwanted male chicks from layer strains of chickens. Recent estimates suggest that at least 280 million such chicks have to be disposed of each year in the European Union alone. The means of disposal used in practice are killing the chicks by maceration, or by gassing or by electrocution (both of the latter followed by incineration). The use of maceration has been recommended as the other can techniques leave approximately 40% of the chicks alive prior to

incineration.

There exists a need, therefore, for simple, accurate methods that can be used at poultry farms that overcome the problems in the prior art and allow for improved animal welfare.

It has now been surprisingly discovered that a W-chromosome specific transcript can be used as the basis for a sex determination method that overcomes the problems previously encountered in this field to date. The W-specific transcript is surprising as it is 3' to 5' in relation to the transcribed strand (5' to 3') for the gene *WPKCI* already known and there is no known Z-chromosome copy.

According to a first aspect of the invention, there is provided a method for the determination of the sex of an avian subject, the method comprising analysing a sample from said subject with a nucleic acid probe comprising an at least 6 base pair fragment from a nucleic acid sequence as shown in Figures 8 to 14.

The present invention provides methods for the determination of the sex of an avian subject, i.e. whether the subject is male or female. The methods of the present invention can be used to determine the sex of a subject of the Class Aves, for example bird species of agricultural importance such as *Gallus gallus* (chicken), turkeys, quail, guinea fowl, commonly referred to as poultry. Such methods may find application in relation to other bird species, such as those bred in captivity, kept as domestic pets, or kept in zoological institutions and examples include penguins, parrots, and rare bird species threatened with extinction, and/or the subjects of breeding programmes for conservation. The subject avian being analysed may be an embryo, a newly hatched chick or a mature adult bird.

Samples that can be assayed according to a method of the present invention include but are not limited to samples of allantois or amnion from the egg, i.e. allantoic fluid

or amniotic fluid, of an avian subject containing a developing embryo; other sources of suitable samples include any convenient sample of a biological nature containing cells, tissue or organs, for example, muscle, heart, brain, lung, liver, chorioallantoic membrane, mesonephrous and blood.

Such methods can be carried out on samples removed from an egg without compromising the viability of the egg using standard procedures. Samples may be removed from the egg manually or by using an automated approach. Machines intended for delivery of vaccine to eggs for incubation can be altered to sample the fluids of the egg in the same manner. The methods can, of course, be performed equally on cultured cells *in vitro*.

The samples to be analysed according to a method of the present invention, may be analysed by means of a DNA amplification procedure, such as the polymerase chain reaction (PCR), or by means of RNA analysis, for example Northern blot, or a Southern blot (Sambrook, J., & Russell, D. W., *"Molecular Cloning"*, Cold Spring Harbor Laboratory Press (2001)), or by using an Invader® RNA Assay (Third Wave – www.twt.com).

The Invader® assay is based on a "perfect-match" enzyme-substrate reaction. Certain endonuclease enzymes are used which recognise and cut only the specific structure formed during the Invader® process. The method relies upon linear amplification of the signal generated, rather than on exponential amplification of the target as in PCR-based approaches. This allows for easy quantification of the target concentration and reduces the effects of sample contamination which may result from exponential target amplification. The system is applicable to analysis of RNA and DNA samples. In the Invader® process, two short DNA probes hybridise to the target to form the structure recognised by the endonuclease enzymes. The enzyme then cuts one of the probes to release a short DNA sequence. Each target can induce the release of several thousand such sequence fragments per hour. Each released sequence fragment can bind to a

fluorescently-labelled probe and form another cleavage structure. When the endonuclease cuts the labelled probe, the probe emits a detectable fluorescent signal. Each released DNA sequence fragment can generate thousands of signals per hour, yielding millions of detectable signals per target (Heisler, L. M. & Lonergan, S. C., *Biomol. Eng. in press*, (2001); Fors *et al Pharmacogenomics*, **1** 219-229 (2000); Heisler *et al Clinical Hemostasis Review*, **14** (11) 10-11 (2000); Leider, K. W. *Advance for Laboratory Managers* 70-71 (February 2000); Treble *et al Gene and Medicine* **4** 68-72 (2000); Leider, K. W. *Advance for Laboratory Managers* 50-52 (November 1999)).

One of the advantages of an Invader® RNA Assay is that it could be carried out in a farm location without complicated equipment or sensitive materials and having no need for specialist experience once initial training has been provided. For example, an Invader® RNA Assay may be devised based upon the 324bp FAF fragment. This could rely on the genomic DNA sequence or on the RNA transcript. The constituents of the assay can be provided dried down, in a multi-well format, such as a 96-well or 384-well plate. In use the currently available Invader® RNA Assay would comprise a probe/Invader® mix (a FAF probe in the present invention), a signal probe, a signal buffer, Cleavase VIII enzyme, a "no target" control, and Rnase-free water. The biological sample (DNA, RNA, amniotic fluid, allantoic fluid, lysed tissue, or lysed blood) can then be added to the wells. The plate can then be incubated in a standard water bath for a defined period and then scanned in a fluorescence reader. The means for detecting the florescence may by a fluorescence resonance energy transfer (FRET) assay.

An RNA-based Invader® assay may be particularly advantageous given the anti-sense nature of the FAF sequence. An RNA Invader® assay would have an oligonucleotide complementary to the transcribed region so would not bind to anything transcribed of the other complementary DNA strand.

The probe to be used in the methods of the invention can be designed according to the general principles of the assay system used. The length of the probe used will depend on whether the assay system is PCR, Northern blot, Southern blot, or an Invader® assay. The probe sequence is at least 6 base pairs (bp) in length and can be any 6bp sequence from a nucleic acid sequence as shown in Figures 8 to 14. This sequence encodes a female specific RNA transcript and can be referred to as "Female Associated Factor" or FAF.

The probe sequence can be from 6 to 10bp, at least 10bp, or at least 15bp, 10bp to 15bp, 15bp to 20bp, at least 20bp, 20bp to 25bp and so on up to at least 324bp. Additional nucleotide residues can be included in the probes designed as required provided that no disruption to the binding of probe to target is seen.

So for a Northern or a Southern assay method, a full-length cDNA probe can be used, or fragments or oligonucleotides based on the full length sequence. For an Invader® assay, probe lengths of from 15 base pairs up to a full length cDNA could be used based on any one of the sequences shown in Figures 8 to 14.

The nucleic acid may be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

Methods of the present invention can determine whether an individual avian subject is male by virtue of the absence of female-specific RNA transcript or DNA sequence, or whether the subject is female by virtue of the presence of the female-specific RNA transcript or DNA sequence in the sample, in which the female-specific RNA transcript, or DNA sequence is derived from a sequence of Figures 8 to 14, or a sequence complementary or homologous thereto.

The percent identity of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues

or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (*i.e.*, % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci. USA* (1990) 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. The NBLAST and XBLAST programs of Altschul et al, *J. Mol. Biol.* (1990) 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al, *Nucleic Acids Res.* (1997) 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, NBLAST) can be used. See www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti *Comput. Appl. Biosci.* (1994) 10:3-5; and FASTA described in Pearson and Lipman *Proc. Natl. Acad. Sci. USA* (1988) 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

A nucleic acid sequence which is complementary to a nucleic acid sequence useful in a method of the present invention is a sequence which hybridises to such a sequence under stringent conditions, or a nucleic acid sequence which is homologous to or would hybridise under stringent conditions to such a sequence but for the degeneracy of the genetic code, or an oligonucleotide sequence specific for any such sequence. The nucleic acid sequences include oligonucleotides composed of nucleotides and also those composed of peptide nucleic acids. Where the nucleic sequence is based on a fragment of the sequences of the invention, the fragment may be at least any ten consecutive nucleotides from the gene, or for example an oligonucleotide composed of from 20, 30, 40, or 50 nucleotides.

Stringent conditions of hybridisation may be characterised by low salt concentrations or high temperature conditions. For example, highly stringent conditions can be defined as being hybridisation to DNA bound to a solid support in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at 65°C, and washing in 0.1xSSC/ 0.1%SDS at 68°C (Ausubel *et al* eds. "*Current Protocols in Molecular Biology*" 1, page 2.10.3, published by Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, (1989)). In some circumstances less stringent conditions may be required. As used in the present application, moderately stringent conditions can be defined as comprising washing in 0.2xSSC/0.1%SDS at 42°C (Ausubel *et al* (1989) *supra*). Hybridisation can also be made more stringent by the addition of increasing amounts of formamide to destabilise the hybrid nucleic acid duplex. Thus particular hybridisation conditions can readily be manipulated, and will generally be selected according to the desired results. In general, convenient hybridisation temperatures in the presence of 50% formamide are 42°C for a probe which is 95 to 100% homologous to the target DNA, 37°C for 90 to 95% homology, and 32°C for 70 to 90% homology.

Examples of preferred nucleic acid sequences for use in a method of the present invention are the sequences of the invention shown in Figures 8 to 14.

Complementary or homologous sequences may be 75%, 80%, 85%, 90%, 95%, 99% similar to such sequences.

The advantages of the methods of the present invention are that the sex of an avian subject can be readily and easily determined based on a single biological sample. There are immediate animal welfare implications in agriculture as the previous practice of whole chick homogenisation can be discontinued.

In a preferred embodiment of the invention there is provided a method for determining the sex of an avian subject, the method comprising the steps of:

(1) obtaining a suitable sample from an avian subject being

(i) an avian embryo *in ovo*; or

(ii) an individual avian

(2) preparing sample for analysis;

(3) probing the sample with a nucleic acid probe based on a sequence of Figures 8 to 14; and

(4) analysing the results of step (3) to determine if individual is male or female.

In a further preferred embodiment of the invention, there is provided a method for determining the sex of an avian embryo, the method comprising the steps of:

(1) obtaining a suitable sample from an avian egg

(2) preparing sample for analysis;

(3) probing the sample with a nucleic acid probe based on a sequence of Figures 8 to 14; and

(4) analysing the results of step (3) to determine if individual is male or female.

In certain embodiments of the invention, the use of the Invader® assay may be preferable. It may also be advantageous to analyse RNA transcripts present in the

sample using the Invader® assay. Alternatively, the Polymerase Chain Reaction (PCR) may be used, either standard PCR and gel analysis, or a quantitative PCR analysis such as Taqman®.

According to a second aspect of the invention, there is provided the use of a nucleic acid sequence or a fragment thereof according to any one of Figures 8 to 14 in a method according to the first aspect of the invention.

According to a third aspect of the invention there is provided a nucleic acid sequence as shown in any one of Figures 8 to 14. Such isolated sequences have use in methods and uses in accordance with the first and second aspects of the invention in determining the sex of an avian subject.

According to a fourth aspect of the invention there is provided a kit of parts comprising a nucleic acid probe comprising an at least 6 base pair fragment from a nucleic acid sequence as shown in any one of Figures 8 to 14 for determining the sex of an avian subject.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

The invention will now be further described by way of reference to the following Examples and Figures which are provided for the purposes of illustration only and are not to be construed as being limiting on the invention. Reference is made to a number of Figures in which:

FIGURE 1 shows the results of Northern analysis of RNA samples from a day 4.5 whole chick embryos using differential display clone 378.2.6 as a probe. Two major bands were detected in females and transcript sizes were approximately 800bp and 1300bp.

FIGURE 2 shows the results of a Southern blot of male and female chicken genomic DNA digested with four different restriction enzymes probed with $^{32}$P-labelled cDNA clone 378.2.6

FIGURE 3 shows the results of W-specific PCR at 57°C, 55°C and 53°C using PCR primers designed from the 796bp sequence of FAF 4.

FIGURE 4 shows the results of a northern blot of total RNA from male and female tissues – heart, brain, lung, liver, chorioallantoic membrane and mesonephrous- probed with $^{32}$P-labelled clone 378.2.6.

FIGURE 5(a) shows the position of the FAF-4 796bp sequence in relation to the *w-pkci* gene. FIGURE 5(b) shows the relative position of the PCR 204bp product with respect to the FAF-4 796bp sequence clone.

FIGURE 6 shows a species blot probed with FAF display fragment for chicken, quail and turkey.

FIGURE 7 shows a diagrammatical representation of sampling of amnion and allantois of a developing chick embryo.

FIGURE 8 shows the nucleotide sequence for the differential display fragment of 324bp (FAF-1).

FIGURE 9 shows the nucleotide sequence of FAF-2 of 796bp.

FIGURE 10 shows the nucleotide sequence of FAF-3 of 772bp.

FIGURE 11 shows the nucleotide sequence of FAF-4 of 796bp.

FIGURE 12 shows the nucleotide sequence of FAF-5 of 1283bp.

FIGURE 13 shows a fragment of the nucleotide sequence of FAF from Turkey.

FIGURE 14 shows a fragment of the nucleotide sequence of FAF from Quail.

Example 1: Differential Display Reverse Transcriptase – Polymerase Chain Reaction (DDRT-PCR)

DDRT-PCR is a powerful molecular tool which allows visualisation of gene expression in any particular cell type or tissue via the creation of RNA fingerprints. Genes which are differentially expressed between two or more samples under study are readily identifiable and recoverable using this technique. Bands representing differentially expressed cDNAs (for example, in male and female tissues) can be recovered and cloned (Miele *et al* In "*Expression Genetics*", eds. McClelland & Pardee, pages 433-444, Natick: Eaton Publishing (1999)(a); Miele *et al Prep. Biochem. Biotech.* **29**(3) 245-255 (1990)(b)). Cloned cDNAs are sequenced and identified following computer-assisted homology searching of the public nucleotide and protein databases. Cloned cDNAs were used for radiolabelling for use as probes in Northern and Southern hybridisation studies according to standard protocols.

Differential display analysis of RNA from male and female whole chicken embryos harvested on days 2.5, 3, 3.5, 4, and 4.5 using primers $dT_{12}$-MC (M=A,G,C) and DM8 (AGTGCCGTTA) revealed two bands which appeared to be female specific. These bands were cut out from the display gel, re-amplified using primers containing EcoR1 restriction sites and cloned into EcoR1 digested pBΠSK$^+$. Colonies obtained were screened for inserts, by PCR, using T7 and T3 primers. Two positive clones, 378.2.2 and 378.2.6 were obtained having insert sizes of approximately 350bp, roughly the size of insert expected from the bands cut from the display gel. A fraction of the

display reactions were run on an agarose/TBE gel and Southern blotted (Miele *et al* 2000). $^{32}$P-labelled inserts from the isolated differential display clones were used to probe the blots. They gave the same female specific banding pattern, confirming that they corresponded to the cDNA bands cut from the display gel. Sequence analysis of the two clones revealed that they were identical.

Northern hybridisation is used to measure the amount and size of RNAs transcribed from eukaryotic genes. After isolating intact mRNA sequences, representing the products of gene transcription, the fragments can be separated and immobilised in a similar way to DNA sequences in Southern hybridisation. Major differences include the need for scrupulous handling to avoid degradation of the RNA by enzymes and the use of denaturing agents such as formamide to preserve the single-stranded, linear nature of the transcripts and allow them to be separated on the basis of their size. (Sambrook, J. & Russell, D. W.,"*Molecular Cloning*: a *Laboratory Manual*" 3rd edition, New York: Cold Spring Harbor Laboratory Press (2001)).

RNA samples from pooled male and pooled female whole chick embryos, days 2.5, 3, 3.5 and 4.5 were used to prepare northern blots. When differential display clone 378.2.2 was used as a probe, two major bands were detected in females. Transcript sizes were approximately 800bp, 1300bp. No bands were apparent in the male samples. The results are shown in Figure 1.

Example 2: Southern Hybridisation

Southern transfer is used to study how genes are organised within genomes using specific probes that hybridise to a portion of the gene. The genomic DNA is digested with restriction enzymes which cut at specific sites and produce a range of fragments of different sizes. The digested DNA is added to wells at one end of an agarose gel. Under the influence of an electric potential the DNA moves down the gel in columns, the fragments becoming separated by size, the smaller fragments moving more

quickly. The DNA is transferred from the gel by blotting onto a solid support, such as a nylon membrane. This is then labelled with the radioactive probe which hybridises to the complementary sequences. These can be visualised as dark bands on a photographic negative in the process of autoradiography. (Sambrook, J. & Russell, D. W., "*Molecular Cloning*: a *Laboratory Manual*" 3rd edition, New York: Cold Spring Harbor Laboratory Press (2001)).

A Southern blot of male and female chicken genomic DNA, digested with four different restriction enzymes, was probed with the $^{32}P$ labelled cDNA clone 378.2.6. A positive signal was seen in the female samples but no bands were detected in male samples even after the blots were overexposed. The results are shown in Figure 2.

Example 3: W-specific PCR

PCR primers were designed from the 796 bp sequence of FAF-4. The sequences of the primers were FAF-Forward primer 5'-AGAATAAACGCCCCTCGATT-3', and FAF reverse primer, 5'-CAGGTTCTCTTTCTCGGTCG-3'. PCR reactions were performed in 25µl 10mM Tris-HCl, 1.5mM MgCl2, 50mM KC1 pH8.3 containing 200µM dNTP's, 0.8µM primers and 1U Taq polymerase. Following an initial denaturation step of 2 minutes at 94°C, DNA was denatured at 94°C for 30 seconds, annealed at 50°C, or 53°C, or 57°C for 30 seconds and extended at 72°C for 30 seconds. Reactions were subjected to 30 cycles of amplification. A final extension step at 72°C for 5 minutes was carried out. After amplification, 20µl of reaction mix was loaded onto a 1% TBE / agarose gel and electrophoresed for 1 hour at 100 volts. The results are shown in Figure 3.

The PCR reaction described in this Example amplifies part of the conserved [324bp]-nucleotide sequence of FAF which is present on the W-chromosome but not the Z-chromosome. Figure 3 shows that for the three annealing temperatures in the region

of the melting temperature of these two primers, there is amplification of the sequence in the female but not male samples. This specificity is maintained even at lower temperatures which increase the possibility of non-specific binding. The results demonstrate that the PCR method can successfully be applied to distinguish unambiguously between male and female DNA.

Example 4: Analysis of expression in day 11 chick tissues

A northern blot of total RNA from male and female muscle, heart, brain, lung, liver, chorioallantoic membrane and mesonephrous was probed with $^{32}$P-labelled clone 378.2.6. The female specific banding patterns obtained were identical in all tissues tested, differing only in the level of expression. The results are shown in Figure 4.

Example 5: Analysis of location of FAF-4 796 bp fragment

Figure 5(a) shows the position of the [FAF]8 796bp sequence in relation to the *w-pkci* gene and Figure 5(b) shows the relative position of the PCR 204bp product with respect to the [FAF]8 796bp sequence clone. The forward primer (A) is

5'-AGAATAAACGCCCCTCGATT-3'

The reverse primer (B) is

5'-CAGGTTCTCTTTCTCGGTCG-3'

Primer details:

| Oligo | Start | Length | Tm | GC% | Any | 3' |
|---|---|---|---|---|---|---|
| Left primer | 414 | 20 | 59.93 | 45.00 | 4.00 | 2.00 |
| Right primer | 617 | 20 | 59.98 | 55.00 | 2.00 | 2.00 |

Example 6: Species blot

The results of a species blot probed with FAF display fragment are shown in Figure 6. The samples probed were obtained from chicken, quail and turkey. Standard genomic DNA extraction from blood from these three species was followed by standard Southern analysis using the original 324bp FAF-1 fragment as a probe.

Example 7: Sampling of amnion and allantois of a developing chick embryo

In order to perform a method of the invention for the purposes of determining the sex of a chick embryo, it is necessary to obtain samples of the amnion and/or allantois of the embryo inside the egg. Small volumes (5µl to 25µl) of amniotic and allantoic fluid can be removed manually or by automated sampling. The collected fluids can then be used as substrates in chick-sexing PCR methods according to the present invention. Sampling of fluids from the chick embryo is shown diagrammatically in Figure 7.

Example 8: Sequencing of differentially expressed RNAs

The sequence of the insert of approximately 350bp found in the two positive clones, 378.2.2 and 378.2.6 identified in Example 1 was sequenced by the method of dideoxy chain termination analysis. The sequence of the FAF-display fragment is shown in Figure 8.

The DNA sequences corresponding to the approximately 800bp and 1300bp RNA bands identified in Example 1 were sequenced as above. The results are shown in Figures 9 to 12 as sequences FAF-2, FAF-3, FAF-4 and FAF-5.

The sequence information obtained was subjected to further analysis to look for homology with other sequences in the available databases and to compare the different sequences with each other.

The differential display fragment of 350bp was found not to match exactly with any of FAF-2, FAF-3, FAF-4, or FAF-5, but a considerable degree of overlap was seen with only relatively few base pair substitutions, or gaps.

In the comparison of the other sequences, FAF-2 was found to show only a 2 nucleotide difference from FAF-5 and FAF-4 when the sequences were aligned. Sequence FAF-2 has a 90% homology with sequence FAF-3 and sequence FAF-4 matches FAF-5 exactly over 796 nucleotides.

In a BLAST 2.2.1 search on www.ncbi.nlm.nih.gov, it was found that FAF-4 has four nucleotide differences with the 5' genomic non-translated region of the *wpcki* gene. The search as a BLAST search of the nr (non-redundant) nucleotide databases.

It is concluded that FAF is located on the complementary strand of the *wpkci* repeat region, in which there are approximately 40 repeats of the *wpcki* gene. However, it lies in the inter-genic region of those repeats with less well conserved sequences. Four FAF transcripts have now been identified and slight differences in the sequence (or in the case of FAF-4 and FAF-5, the different lengths) suggests that they each come from a different repeat of the gene.

CLAIMS

1. A method for the determination of the sex of an avian subject, the method comprising analysing a sample from said subject with a nucleic acid probe comprising an at least 6 base pair fragment from a nucleic acid sequence as shown in Figures 8 to 12.

2. A method as claimed in claim 1 in which the avian is a member of Class Aves

3. A method as claimed in claim 3, in which the avian is selected from the group consisting of *Gallus gallus* (chicken), turkey, quail and guinea fowl.

4. A method as claimed in any one of claims 1 to 3, in which the sample is allantoic fluid or amniotic fluid

5. A method as claimed in any preceding claim, in which the sample is taken from an egg.

6. A method as claimed in any preceding claim, in which the analysis of the sample comprises a nucleic acid amplification procedure

7. A method as claimed in claim 6, in which the nucleic acid amplification procedure is the polymerase chain reaction (PCR) or an Invader® assay.

8. A method as claimed in claim 7, in which the assay is an Invader® assay.

9. A method as claimed in claim 8, in which the assay comprises amplification of RNA in the sample.

10. The use of a nucleic acid sequence or a fragment thereof according to any one

of Figures 8 to 12 in a method according to any one of claims 1 to 9.

11. A nucleic acid sequence as shown in any one of Figures 8 to 12.

12. A kit of parts comprising a nucleic acid probe comprising an at least 6 base pair fragment from a nucleic acid sequence as shown in Figures 8 to 12 for determining the sex of an avian subject.

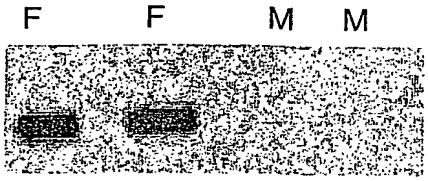



Figure 1 Day 4.5 whole embryo

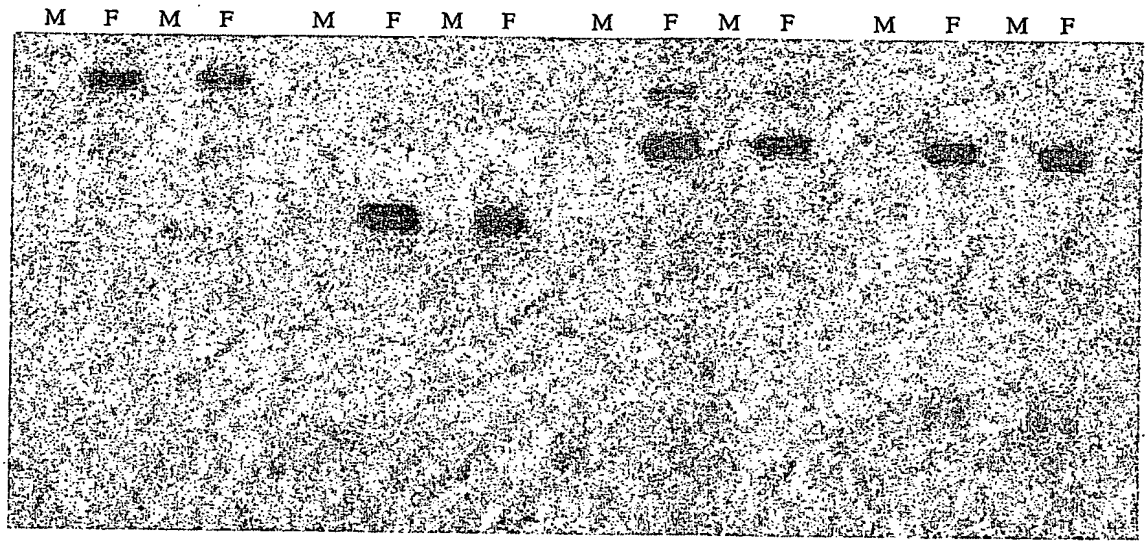



Figure 2 Southern analysis

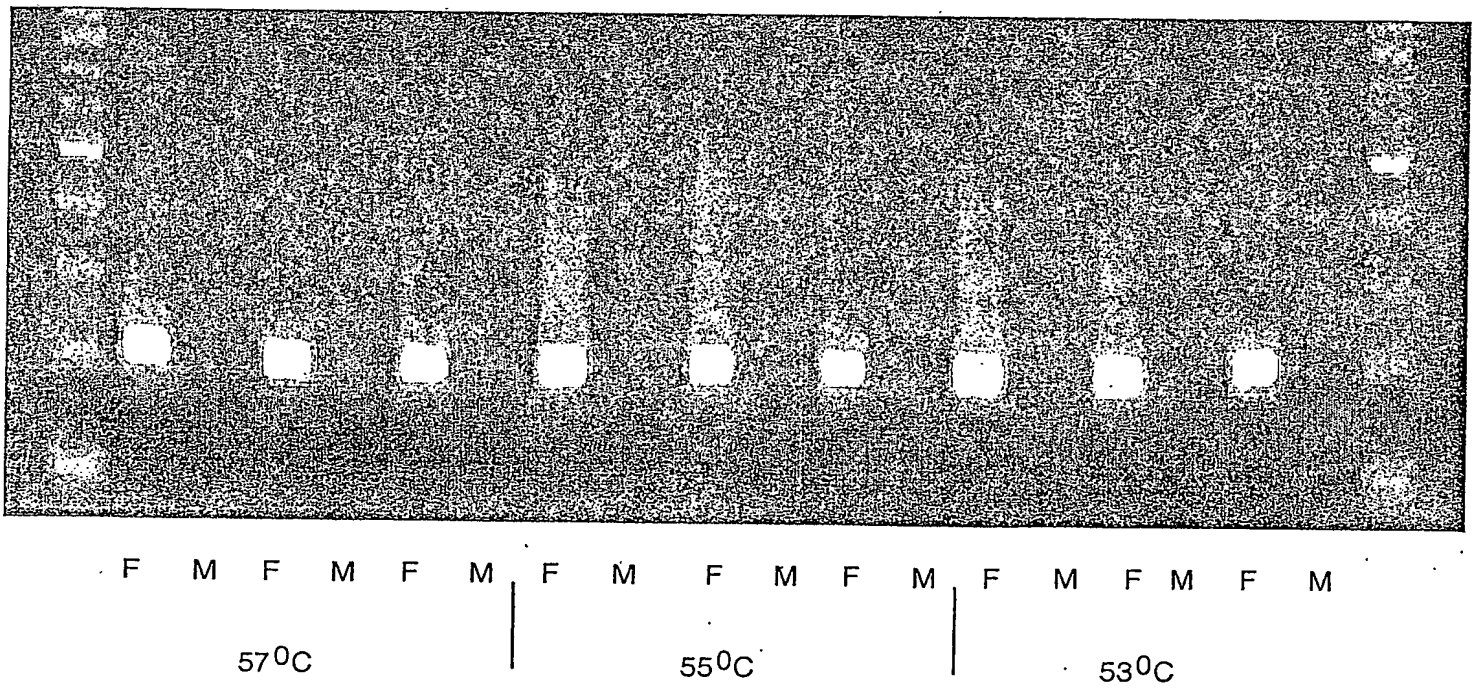



Figure 3 W-specific PCR

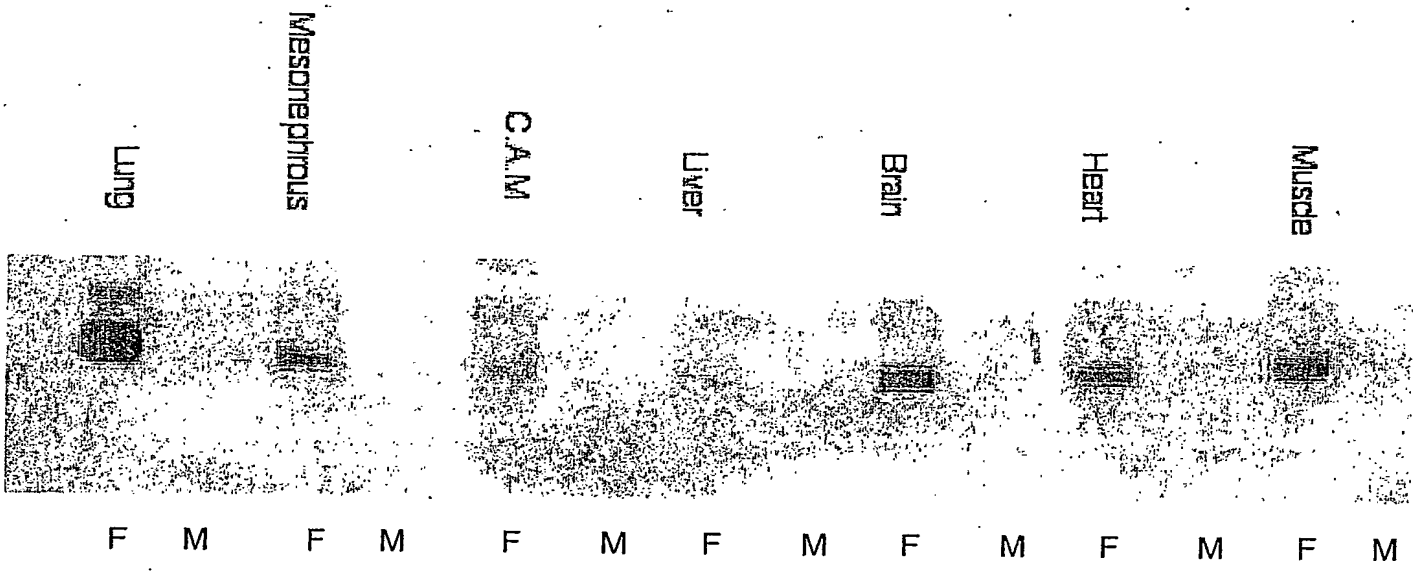
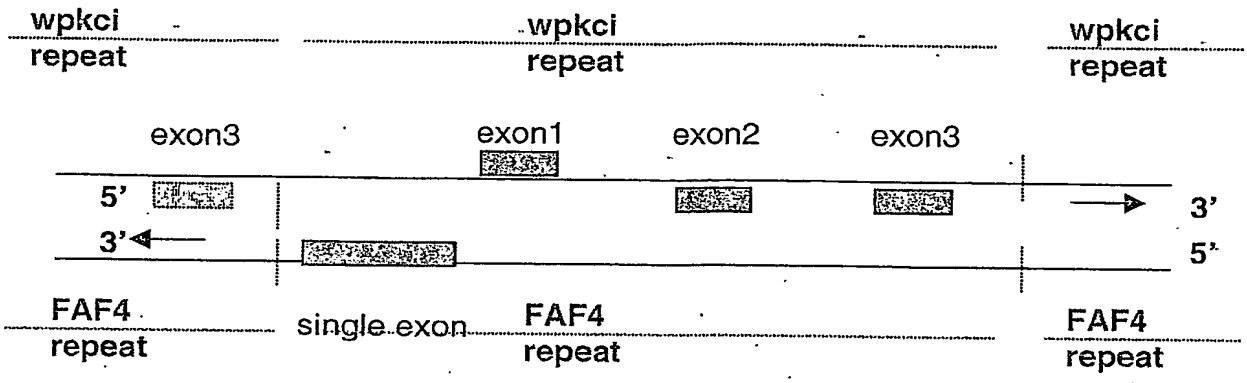



Figure 4 Northern analysis of FAF mRNA expression in the tissues of developing chicken embryos at day 11.5: lung, mesonephros, chorioallantoic membrane (CAM), liver, brain, heart and muscle isolated from male (M) and female (F) embryos

**Position of the FAF4 796 bp sequence in relation to the w-pkci gene**




```
Forward primer (A) = AGAATAAACGCCCCTCGATT
Reverse primer (B) = CAGGTTCTCTTTCTCGGTCG
```

Figure 5(a) Female-specific PCR primers

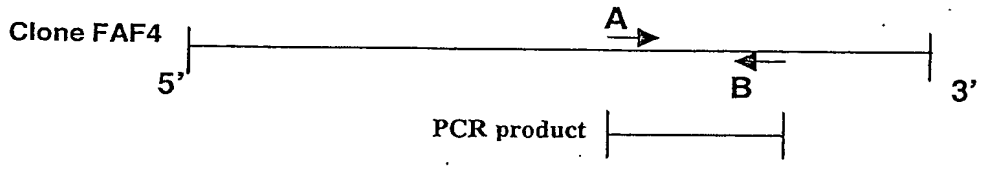



Figure 5(b) Relative position of the PCR 204 bp product with respect to the FAF4 796 bp sequence clone

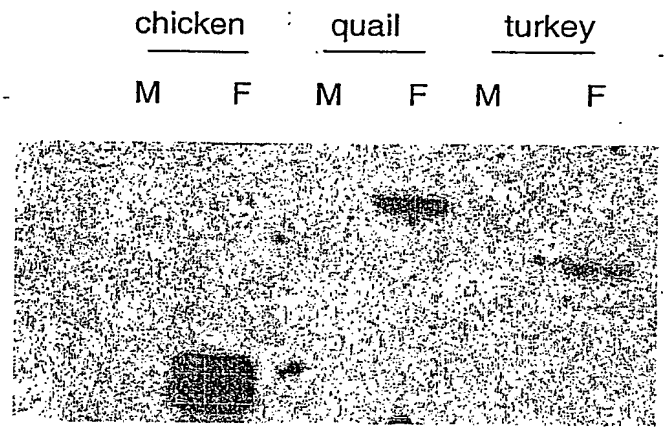



Figure 6 Species blot

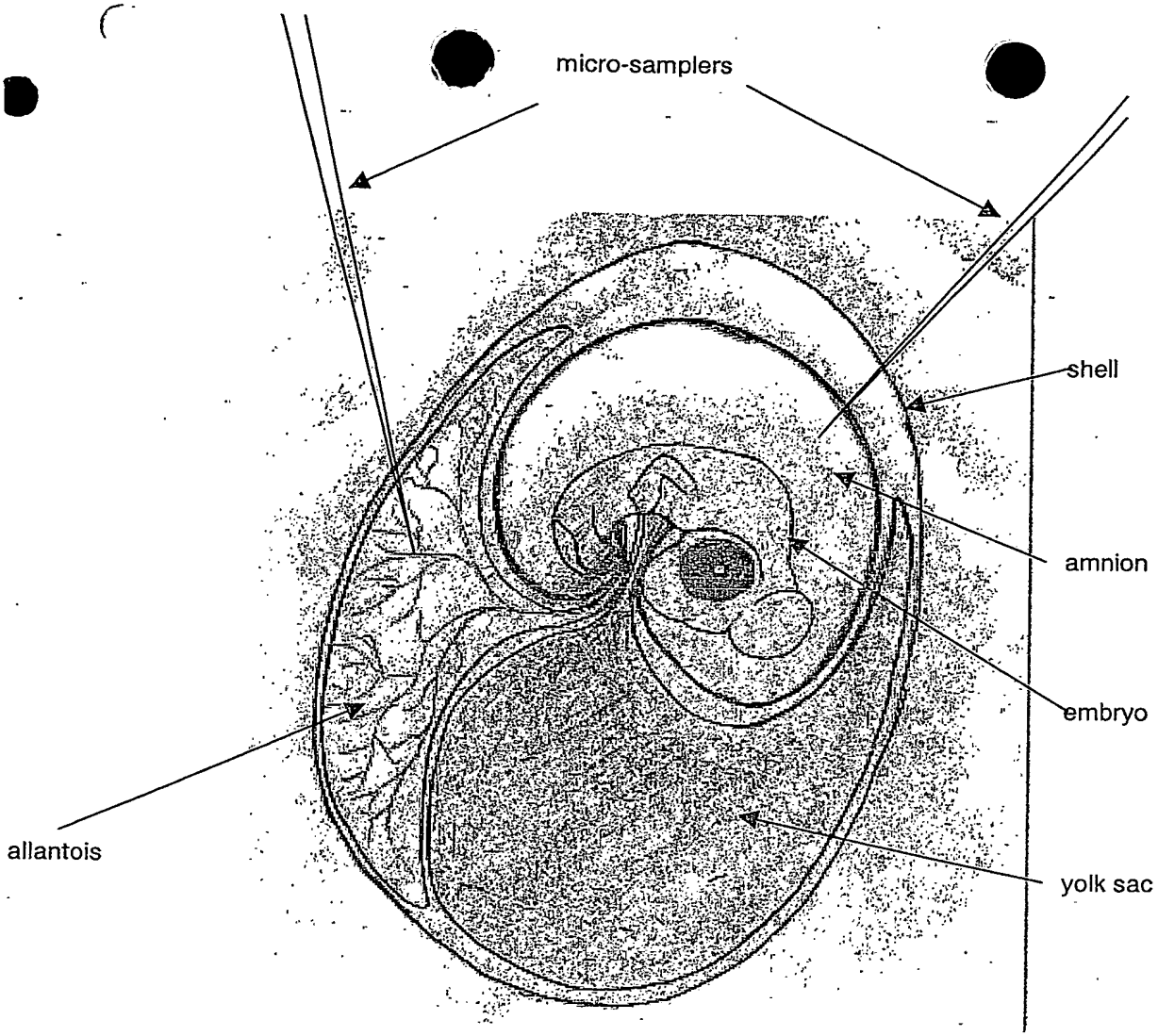



Figure 7

**FAF1**

```
   1  AGTGCCGTTA CTATGAGCAA CCCAAGGAGA ACCAGACAGT ATATATATAT
  51  GTGTATGACT CTGCAAAACC TTTGTAGCGC GCATTTTCCC TTGCTGTGTT
 101  TTCCTTCCGC CTGTGATCGA CCGAGAAAGA GAACCTGCCC CTCTACCCCT
 151  GCTTCCAACC AGAATCATGA AACACTGTCA CACTGCGGTG GTAACCATCT
 201  CTGCATTCCT GTAACAAATC CTTGCTTTTC TTTCTGTCTT TTTACTATTG
 251  CTTTCGTCAT CCCACCTCCC ATCCCCCGGC CTAGCTAACC AAAACTTTCT
 301  ACAATAAACC GGTTGGGC
```

Figure 8

**FAF2**

```
   1  GGCGCTGGGG GCTTTTTGGT GCCGATCCCT CCCGTCAAAT GGCCGTCAAA
  51  TGTTGACGGG GCAGGCCAGG AGTTTGCCAT CTTTGCATGA AGGGACAGGC
 101  AACTCGGGGA GAGTGCAAGG ATGTTGCTAG CATGCGCAGG GAGAAAATTC
 151  GACAGGCCAA AGCCCAGCAC GACCTTAATA TGGCCGCCAT TGTTTGAGAT
 201  GATTAAAACT ATGTTTTTAC GAACATATTA ATAAGAGCAA GAGGAGGGCC
 251  AAGGAGAATC TCCCTTCTTT ATTCAACGCG GTGGGGAACA TCACCATCGA
 301  GGAGGAGGGA AAGGCTGAAG TTCCCAACGC CTTCTTCACT TCTGGCTTTA
 351  GCAGTGAGAC CTGCTATCCC CAGGGTACTC AGCCCCCTGA GCTGGAAGAC
 401  GGGGCCGGGG AGCAGAATAA ACGCCCCTCG ATTCCCAGTG CCTTCTTTAC
 451  TTCTGTCTGT TTCTGACTGT TGCACCTGTG CTGGACGTGC CGTTACTATG
 501  AGTAACCCAA GGAGAACCGG ACAGTATATA TATGTATGGA CCCTGCAAAA
 551  ACTTTGCGCG CGCTTTTCCC TTGTTGTGTT TTCCTTCCGC CTGTGATCGA
 601  CCGAGAAAGA GAACCCGCCC CCCCCCCGCT TCCAACCGGA ATCATGAAAC
 651  ATTGTCACAC TGCGGTGGTA ACCATCTCTG CATTCCTGTA ACAAATCCTT
 701  GCTTTTCTTT TCTGTCTTTT CACTATTGCT TTCGTCATCC CACCTCCCAT
 751  CCCCAGGCCT AGCTAACCAA AACGTTTTAC AATAAACCGG TTGGGC
```

Figure 9

**FAF3**

```
   1  CGGTCAAATG GCCGTCAAAT GTTGGCGGGG CAGGCCAGGA GTTTGCCATC
  51  TTTGGATGAA GGACGGGCAA CTCGGGGAGA GTGCCAGGAT GTTGCTAGCA
 101  TGCGCAGGGA GAAAATTCGA CAAGCCAAAG CCCAGCAAGA CCTTAATCTG
 151  GCCGCCATTG TTCGAGATGA TTAAAACAAT GTTTTTACGA ACGTATTAGT
 201  AGCAAGAGGA GGGCCAAGGA GAATCTCCCT TCTTTATTCG ACGCGGTGGG
 251  GAACATCACC ACCGAGGAGG AGGAAAAGGC TGAAGTTCTC AACGCCTTCT
 301  TCACTTCTGT CTTTAGCAGT GAGACCAGCT ATTCTCAGGG TACTCAGCCC
 351  CCTGAGCTGG AAGACGGGGC CGGGGAGCAG AATAAACGCC CCTCAATTCC
 401  CAGTGCCTTC TTTACTTCTG TCTGTTCTGA CTGTTGCACC GGTGCTGGAC
 451  GTGCCGTTAC TATGAGCAAC CCAAGGAGAA CCAGACAGTA TAGATATATA
 501  TATATGTATG GACTCTGCAA AAACTTTTGT GCGCGCTTTT CCCTTGCTGT
 551  GTTTTCCTTC CGCCTGTGAT CGACCGAGAA AGAGAACCTG CCCCCCCACC
 601  CCTGCTTCCA ACCAGAATCG TGAAACATTG TCACACTGCG GTGGTAACCA
 651  TCTCTGCATT CCTGTAACAA ATCCTTGCTT TTCTTTTCTG TCTTTTCACT
 701  ATTGCTTTCG TCATCCCGCC TCCCATCCCC AGGCCTAGCT AACCAAAACT
 751  TTCTACAATA AACCGGTTGG GC
```

Figure 10

FAF4

```
   1  GGCGCTGGGG GCTTTTTGGT GCCGATCCCT CCCGTCAAAT GGCCGTCAAA
  51  TGTTGACGGG GCAGGCCAGG AGTTTGCCAT CTTTGCATGA AGGGACAGGC
 101  AACTCGGGGA GAGTGCAAGG ATGTTGCTAG CATGCGCAGG GAGAAAATTC
 151  GACAGGCCAA AGCCCAGCAC GACCTTAATA TGGCCGCCAT TGTTTGAGAT
 201  GATTAAAACT ATGTTTTTAC GAACATATTA ATAAGAGCAA GAGGAGGGCC
 251  AAGGAGAATC TCCCTTCTTT ATTCAACGCG GTGGGGAACA TCACCATCGA
 301  GGAGGAGGGA AAGGCTGAAG TTCCCAACGC CTTCTTCACT TCTGGCTTTA
 351  GCAGTGAGAC CTGCTATCCC CAGGGTACTC AGCCCCCTGA GCTGGAAGAC
 401  GGGGCCGGGG AGCAGAATAA ACGCCCCTCG ATTCCCAGTG CCTTCTTTAC
 451  TTCTGTCTGT TTCTGACTGT TGCACCTGTG CTGGACGTGC CGTTACTATG
 501  AGTAACCCAA GGAGAACCGG ACAGTATATA TATGTATGGA CTCTGCAAAA
 551  ACTTTGCGCG CGCTTTTCCC TTGTTGTGTT TTCCTTCCGC CTGTGATCGA
 601  CCGAGAAAGA GAACCTGCCC CCCCCCCGCT TCCAACCGGA ATCATGAAAC
 651  ATTGTCACAC TGCGGTGGTA ACCATCTCTG CATTCCTGTA ACAAATCCTT
 701  GCTTTTCTTT TCTGTCTTTT CACTATTGCT TTCGTCATCC CACCTCCCAT
 751  CCCCAGGCCT AGCTAACCAA AACGTTTTAC AATAAACCGG TTGGGC
```

Figure 11

FAF5

```
   1  CGCAACGGGC GCTCGTTCCA GAGGGCCTGC GAGCGCGCTA GGGTGGGGGA
  51  GGGGTGGGAC GGGAGGGCAA GGGAAGAATC GCGCGACGCG CAGCAAAGCC
 101  GCGGCTACCT CCTCGTCCAC AACGGCTCCT CCTCGCGGAT AACGTTGGCG
 151  GAGAACTCCT GGCGGGCGAC TTTTCCCAAG AGAGCGGCGC CACCGCGCCA
 201  GGCGGCCGGC GACCTAACGA TCCCGCCGGC CATGACGGCG CCCGCTCGCT
 251  ACAACACTCC CTCAGCCCCA AACCTCCCCA GCACGGCTCA GCATGGCTCA
 301  GCACGGCTCG GCTCGCCTCG GCTCGCCTCG GCCCGGTCCC GCCCTCGGCG
 351  GCGCTCATTG GGCCGACAGA GCGCCGCGGC CGTTTCCGCG CCTCGGTTGG
 401  CTGTCTCGCC TGCCCTTTAA GCTTGTCCCC GCCCTGTAGG CGGCTCCGCT
 451  CCCGTCGGCC CGGTGCTTAT CGGGGCTCAG GGACTTAGGC GCTGGGGGCT
 501  TTTTGGTGCC GATCCCTCCC GTCAAATGGC CGTCAAATGT TGACGGGGCA
 551  GGCCAGGAGT TTGCCATCTT TGCATGAAGG GACAGGCAAC TCGGGGAGAG
 601  TGCAAGGATG TTGCTAGCAT GCGCAGGGAG AAAATTCGAC AGGCCAAAGC
 651  CCAGCACGAC CTTAATATGG CCGCCATTGT TTGAGATGAT TAAAACTATG
 701  TTTTTACGAA CATATTAATA AGAGCAAGAG GAGGGCCAAG GAGAATCTCC
 751  CTTCTTTATT CAACGCGGTG GGGAACATCA CCATCGAGGA GGAGGGAAAG
 801  GCTGAAGTTC CCAACGCCTT CTTCACTTCT GGCTTTAGCA GTGAGACCTG
 851  CTATCCCCAG GGTACTCAGC CCCCTGAGCT GGAAGACGGG GCCGGGGAGC
 901  AGAATAAACG CCCCTCGATT CCCAGTGCCT TCTTTACTTC TGTCTGTTTC
 951  TGACTGTTGC ACCTGTGCTG GACGTGCCGT TACTATGAGT AACCCAAGGA
1001  GAACCGGACA GTATATATAT GTATGGACTC TGCAAAAACT TTGCGCGCGC
1051  TTTTCCCTTG TTGTGTTTTC CTTCCGCCTG TGATCGACCG AGAAAGAGAA
1101  CCTGCCCCCC CCCCGCTTCC AACCGGAATC ATGAAACATT GTCACACTGC
1151  GGTGGTAACC ATCTCTGCAT TCCTGTAACA AATCCTTGCT TTTCTTTTCT
1201  GTCTTTTCAC TATTGCTTTC GTCATCCCAC CTCCCATCCC CAGGCCTAGC
1251  TAACCAAAAC GTTTTACAAT AAACCGGTTG GGC
```

Figure 12

**TURKEY FAF REGION**

```
   1  TGCCGTTACT ATGAGCAACC CAAGGAGAGC CAGACAGTGT ATATATGTAT
  51  GGACTCTGCA AAAACTTTGT GCGCGCTATT CCCTTGTTGT GTTTTCCTTC
 101  CGCCTGTGAT CGACCGAGAA AGAGAACCTG CACCCCCCAG CCCCGCTGCC
 151  AACCAGACTC ATGAAACATT GTGACACTGC GGTGGTAACA ATCTCTGCCT
 201  TCCTGTAACA AATCCTCGCT TTTCTTTTCT GTCTTTTTAC TATTGCTTTC
 251  TTCGTCCCAC CTCCCATCCC CAGGCCTAGC TAACC
```

Figure 13

**QUAIL FAF REGION**

```
   1  ACTAGTGATT GCCGTTACTA TGAGCAACCC AAACAGTGGA CAGTGTATAT
  51  ATAAGGGCTG CAAAAATAAG AGCATATGAT TTCCCTTGTA TTTTCCTTCT
 101  GCCTGTGATC GGCCAAGAAA GAGGGAGAGA ATTGACAGCC TGCACTGCCT
 151  CTGCTGACCA GACTCATGGA ACACTGTCAT ACTGCAGTGA TAACTATCTC
 201  TGCATTCCTA TAACAAACCC TTGCTTTTAT TTTCTTTCTT TTTACTATCA
 251  TTTTCTTCAT CCCACCTCCT GTCCCCAGGC CTAGCTAACC AATC
```

Figure 14